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13. ABSTRACT *(Maximum 200 Words)*

Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis-drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem. Recognizing this emerging crisis, it is necessary to identify new strategies for the identification and development of new antimalarials. The goal of this work is the development of a framework for antimalarial drug development into the 21st century.

A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs based on an understanding of the parasite biology are key elements in this new strategy. Clearly the development of a new antimalarial will require both basic and applied research working in concert with one another.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. During the preceding period, the research has focused on the two objectives, namely the analysis of critical genes in the *Plasmodium falciparum* for their role in drug resistance and as potential new drug targets using both the homologous P. falciparum system and the heterologous yeast system. We have initiated experiments during this grant period which take an alternate technical approach to achieve the goals in our statement of work and represent applications of new technology which did not exist at the time of our original planning process. These include the analysis of gene expression in response to drug treatment using the method of Serial Analysis of Gene Expression and the use of DNA Chip technology in the analysis of the yeast heterologous system. These approaches complement ongoing work and will provide us with new insights into drug resistance and provide excellent tools for the identification of potential new drug targets.

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(5) Introduction

Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis-drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem. Recognizing this emerging crisis, it is necessary to identify new strategies for the identification and development of new antimalarials. The goal of this work is the development of a framework for antimalarial drug development into the 21st century.

A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs based on an understanding of the parasite biology are key elements in this new strategy. Clearly the development of a new antimalarial will require both basic and applied research working in concert with one another.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed, one the development and characterization of a homologous transformation system and two the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with goal of using this information in the identification and development of new antimalarial drugs. The development of these tools should facilitate future drug development and allow us to translate our molecular genetic knowledge into the practical identification and development of new antimalarials. This is a new strategy and it is being applied because of the crisis facing us in antimalarial drugs. The previous strategy, namely lead directed screening must be supplemented by new strategies or we will be faced with multiresistant *Plasmodium falciparum* and no drugs to treat it.

Malaria represents a major and increasing threat to the U.S. Military. Many of the sites of current or potential U.S. Military involvement are endemic for malaria and in several sites, multidrug resistant *P. falciparum* represents a major problem especially for non-immune military personnel. Current drugs available to the U.S. Military are quickly losing their effectiveness because of emerging and spreading drug resistance. This work is directed both at identifying new drugs and drug targets, but equally importantly toward an understanding of drug resistance mechanisms with the goal of preventing or overcoming drug resistance in the malaria parasite.

(6) Body

During the preceding period, the research has focused on the two objectives, namely the analysis of critical genes in the Plasmodium falciparum for their role in drug resistance and as potential new drug targets using both the homologous *P. falciparum* system and the heterologous yeast system. We have initiated experiments during this grant period which take a alternate technical approach to achieve the goals in our statement of work and represent applications of new technology which did not exist at the time of our original planning process. These include the analysis of gene expression in response to drug treatment using the method of Serial Analysis of Gene Expression and the use of DNA Chip technology in the analysis of the yeast heterologous system. These approaches complement ongoing work and will provide us with new insights into drug resistance and provide excellent tools for the identification of potential new drug targets. Summaries of the ongoing work, including recent data are included for each of the projects.

A. Functional analysis of putative drug resistance genes and new drug target genes in the malaria parasite through the further development of a transformation system for the malaria parasite including:

1. Development of methods to express and modify parasite genes
2. Development of methods for targeted gene disruption

The overall goal of this work is to understand gene expression the parasite, in particular, the expression of genes critical for drug response and resistance. This work will also lead to the development of methods to identify critical genes as future drug targets. One of the key obstacles hindering our progress in this work is a fundamental understanding of gene expression in the parasite and this has limited our ability to manipulate the organism. Another obstacle had been the limited number of genes that had been examined in the parasite. Progress in the Plasmodium falciparum genome project and development of new technology has provided an opportunity to overcome these obstacles in the parasite. We have now initiated a project to analyze gene expression in Plasmodium falciparum using the newly developed method of Serial Analysis of Gene Expression. This work is being done in close collaboration with Dr. Keith Martin, WRAIR.

(i) Serial Analysis of Gene Expression in Plasmodium falciparum

The malarial parasite exhibits a complex life-cycle in two vastly different hosts, mammalian and insect, and is able to invade and survive in cells as different as hepatic cells, erythrocytes and mosquito mid-gut cells. Understanding the parasite's ability to interact with these cell types, evade the host immune system and adapt rapidly to chemotherapeutic drugs will yield valuable information on the control of a disease that affects many countries of the developing world. One approach to understanding these different aspects of Plasmodium biology is to study responses of the parasite to different growth conditions at the level of gene expression.

In recent years, numerous techniques have been developed for the analysis of gene expression under different growth conditions; these include differential display (Liang and Pardee 1992), serial analysis of gene expression (SAGE) (Velculescu et al. 1995), micro-arrays (Lashkari et al. 1997) and DNA chip technology (Johnston 1998). For Plasmodium species, micro-arrays and DNA chips are presently unavailable while differential display may not be sensitive or quantitative enough to detect minor changes in RNA levels. Hence, we propose to use SAGE technology as a means to detect subtle changes in gene expression in the malarial parasite.

SAGE relies on 2 basic premises: first, a short (9-14bp) sequence tag, derived from cDNA, is sufficient to uniquely identify genes and second, quantitation of the relative occurrence of such tags can give an accurate representation of levels of gene expression. The SAGE procedure is outlined in Figure 1. Briefly, SAGE involves the synthesis of cDNA from poly-adenylated RNA. The cDNA is digested with a restriction enzyme (anchoring enzyme, AE) that cuts with a high enough frequency to target a majority of cDNA molecules in the population. The choice of the AE will determine whether all messages are adequately represented. Next, linkers that carry a recognition site for a type IIS restriction enzyme (tagging enzyme, TE) are ligated to the cDNA ends. The type IIS enzyme will cleave DNA 9-16bp away from its recognition site, generating short tags. These tags are amplified by PCR, purified and ligated into concatemers that are cloned into a plasmid vector. Sequencing of the plasmids results in identification and quantification of the SAGE tags.

SAGE has been successfully adapted for analysis of the malarial parasite in this lab. *P. falciparum* (strain 3D7) was grown under standard culturing conditions. Two sets of trophozoite stage parasites (1010), matched for growth conditions, were treated with either no drug or 50nM chloroquine for 6 hours. Total RNA from the two pools of parasites was selected on oligo-dT cellulose to yield poly-A+ RNA, 5 micrograms of which were used to synthesize cDNA. The cDNA was digested with the restriction enzyme NlaIII (AE) that is predicted to digest *P. falciparum* genomic DNA every 400-500bp at CATG sites (sequence analysis at TIGR and Sanger centers). Figure 2 shows that NlaIII digestion of 3D7 cDNA results in a collapse of the smear to an average size of 500bp, as expected. As *P. falciparum* is predicted to contain one open reading frame every 4-5 kilobases (TIGR and Sanger sequence analysis), digestion with NlaIII should result in a highly representative SAGE library.

In the next step, the digested cDNA was divided into two pools and ligated to linkers A and B, containing a recognition site for the type IIS enzyme BsmFI as well as binding sites for PCR primers. Digestion with BsmFI yields molecules of approximately 50bp (40bp linker + 10bp cDNA tag) that are blunt-ended with Klenow polymerase. Incorporation of radiolabeled dCTP into the blunt-end reaction enables the visualization of these 50bp molecules (shown in Figure 3). The two pools of blunt-ended DNA were ligated together resulting in structures of ~100bp; these were then PCR-amplified using linker-specific primers (Figure 4). Purified 100bp molecules were digested with NlaIII to release 26bp ditags (shown in Figure 5), and the ditags were ligated into concatemers that

were cloned into the SphI site of the pZero vector. Clones containing large inserts (greater than 200bp) were identified by colony PCR and then sequenced (Walter Reed Army Institute, Harvard Medical School, TIGR). Sequence data was analyzed using SAGE software (Genzyme) which extracted tag counts as well as compared experimental tag data to Genbank-format sequence databases.

From the control population, we have obtained approximately 600 clones containing an average of 25 tags per clone. Hence we expect the present experiment to yield a total of 15,000 tags. In yeast, an organism that has a similar genome size as well as a comparable number of ORFs, roughly 20,000 tags were sufficient to define the entire repertoire of genes expressed during log phase growth (Velculescu et al. 1997). Currently, data has been obtained from 2300 tags; the remaining tags are being sequenced. A small fraction of the available tags were used to search the *P. falciparum* sequence databases by BLAST analysis: 48% gave unique matches while 45% did not match Plasmodium sequences. These results are consistent with the fact that only 60% of the *P. falciparum* genome has been sequenced to date. The rest of the tags (7%) matched to multiple genes; this percentage is comparable to that obtained during SAGE analysis of human pancreatic cells (5%)(Velculescu et al. 1995). Thus, although the genome of *P. falciparum* is 70-80% A-T rich and therefore considerably less complex than yeast or mammalian genomes, 14bp tags are sufficient to identify unique genes.

Abundant tags matched to housekeeping genes such as cytochrome oxidase and signal transduction genes such as ras-related nuclear protein, MAP-2 kinase, etc. We also detected transcripts encoding cell surface molecules (rifins, PfEMP-1) and genes of unknown function (cg6). Some abundant tags matched Plasmodium sequences that had not previously been shown to contain any open reading frames. Therefore, this work will be useful in assigned ORFs to sequence reads generated by the *P. falciparum* Genome Project.

Now that a baseline SAGE profile has been established in the 3D7 strain, a SAGE library of chloroquine-treated parasites is currently being generated.

(ii) Analysis of Gene Expression in Plasmodium falciaprum

As the work on the global analysis of gene expression is ongoing, we have continued our efforts to understand gene expression at the level of the individual gene in order to develop methods to modify expression using molecular genetics. Again, with the additional information provided by the genome project, we have been able to make excellent progress on this work and have made a preliminary observation which indicate that we may be able to readily identify cis-acting elements which control gene expression through an analysis of comparative genomics.

5' Untranslated Region sequence variation in strains of *Plasmodium falciparum*.

Transcriptional regulation has not been well defined in *Plasmodium falciparum*. A review of studies that have looked at the regulation of different genes indicate that regulation in the parasite may be different from the classic model of regulation in eukaryotes. While the mechanism may be different, it is likely that transcriptional regulation plays an important role in genes such as *pfmdr1*. This is supported by the evidence in yeast, where *PDR* genes are transcriptionally regulated. Thus in an effort to further characterize the role of *pfmdr1* in drug resistance, we have begun to map the 5'untranslated region of the gene.

A contig on chromosome 5 containing 3D7 strain *pfmdr1* coding and noncoding sequence has been extracted from the *Plasmodium falciparum* genome database. The coding region of this contig has 100% identity with the coding sequence of the D10 strain *pfmdr1* clone in Genbank (gi:9935). However, the 5'UTR of the genes only has 60% identity. This reduction in identity occurs in a pattern of complete homology, followed by minor variation, followed by major variation at the most upstream end of the 3D7 and D10 sequence. This pattern may be indicative of selective pressure in the gene.

We were interested to see if this pattern of increasing variation was present in other *P.falciparum* genes. A comparison of upstream sequence of the 3D7 and T9/96 (gi: 160127) *P. falciparum* calmodulin genes revealed a similar pattern. Of course, the possibility of cloning artifacts and sequencing errors must be ruled out before the significance of this result can be determined. Both sets of sequences were aligned utilizing the Clustal W alignment tool. They were anchored at the putative translational start site, and matched in length to minimize gaps. 3D7, the reference strain, is highlighted in bold for both alignments.

SEQUENCE ALIGNMENT OF 3D7 AND D10 *pfmdr1* 5'UTR.

```
80.5% identity in 503 nt overlap; score: 1217

        10        20        30        40        50        60
_       TAAAATATACATAATTAAATATAAAAATGACATTATATTTTTGTTAAATTATACAGAAGA
        ::::   :  ::::  :::::::: ::: :     : :::::  ::::: ::   :   :
_       TAAATCTTTTATAA--AAATATAATAATTAATAATTTTTTTTAATAAATAATTTTGTTTA
             10        20        30        40        50        60

        70        80        90       100       110       120
_       AAAAAAAAAAAAAAAAAAAATAGAAGTAAATTGTATAGAATTATTTNTTTATTAATATTAT
        ::  :: :: :   ::   ::  : : : :: :  : : :: :::.: ::::  :::
_       AATTAATAATATGTAATTTTATTATTTATTTATTAACATTTTTTTTTATATTT-TATATA
               70        80        90       100       110       120

      130       140       150       160       170       180
_       TATTTTATTTTGAATAA--AACTATTTTNGTATCTAATAATAAATA-TAATAACACATAT
         : :  :: :  :::::  :: : : : .: :: ::: ::::  :: ::::   :  :::
_       AAATACATATATAATAACTAAATTTATGCGCATATAAAAATATCTAATAATTTTAATTAT
               130       140       150       160       170       180

        190       200       210       220       230       240
_       ATATATATATATAT-ATATATATATATATTATT---TNANTTATTATTATATTTTTTTTT
        :::::: ::::::: ::: :::::  ::::::    :.:. ::::::::::::: : :: :
_       ATATAT-TATATATTATACATATAATTATTATAACGTTATATATTATTATATTATATTAT
               190       200       210       220       230

             250       260       270       280       290       300
_       TTATTATTTTTTTTGTCATTGTGTAAATATATAAATATATATANTATATATATTATTA
        : ::::::::::::::::::::::::::::::::::::::::::.:::::::::::::::::
_       T-ATTATTTTTTTTGTCATTGTGTAAATATATAAATATATATATTATATATATTATTA
      240       250       260       270       280       290

             310       320       330       340       350
_       TTTCAACATTGTTTATATATATATATATATATATATATATATATATATATTG
        :::::::::::::::::::::::::::::::::::::::::    ::::::::::::::::
_       TTTCAACATTGTTTATATATATATATATATATATATATATATATTTATATTTATATATTG
      300       310       320       330       340       350

      360       370       380       390       400       410
_       ATATATGTGTACATAGCTTATTTCATTTATAAGATTTAGATTTTGTTTTTAATATTATAT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_       ATATATGTGTACATAGCTTATTTCATTTATAAGATTTAGATTTTGTTTTTAATATTATAT
      360       370       380       390       400       410

      420       430       440       450       460       470
_       AATTTTGTTTGTTACAAATTAATTAATTATTTCTTTATTTCTTTATTTTATTTACATTTT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_       AATTTTGTTTGTTACAAATTAATTAATTATTTCTTTATTTCTTTATTTTATTTACATTTT
      420       430       440       450       460       470
```

Sequence alignment of 3D7 (TOP) and T9/96 (BOTTOM) Calmodulin 5'UTR.

76.2% identity in 1015 nt overlap; score: 2163

```
      60        70        80        90       100       110
_     AAATATTATTTATAACAAGAGAAAAGGCAGAAACAAAATAA-ATTATAATAAAAAACACA
      ::  ::: : :: :: ::   ::::   :   : : :::: ::: :: :  :  : : :
_     AACCATTTTGTAAAAAAAATTAAAATATATTTATATAATATTATTTTATTTTATTATATA
            80        90       100       110       120       130

     120       130       140       150       160       170
_     TTTTTTTATATTTGTATGAATATATTTTTTGTTATGCCTAAAAAAAAAATAGGATTATC-A
      :: : :::: ::: : :  :: : : :::: :: :  ::: :::          :::::
_     TTATATTATTTTTATTTTTATTTTTATTTTTTTTTCTCTACAAATT-------TTATCTA
           140       150       160       170               180

      180       190       200       210       220           230
_     TATTTTTATATAAAATGTAAGGATTTCAAAATATATATAATTT---TTTAAAATAACAAA
      :   :::::   :::  ::   ::::: ::  ::: ::::::    : : :: : : : :
_     TTGGTTTATTATAAAAATATCTATTTCTAATAATAAATAATTAAGATATCAATTTATAGA
        190       200       210       220       230       240

        240       250       260       270       280       290
_     AAGGGAACATTTTTTTTTTTTTTTTTAACATTTTCATGCCACGTTGACAAGAATTTTTAA
      ::   :: :: :  :: : :  :::::   ::::      :   : :::   ::  :::
_     AACAAAATATATACTTGTATAATTTTATTTTTTTATATAAATCATTACATATATAATTAT
        250       260       270       280       290       300

        300       310       320       330       340       350
_     AAAATCCATTAAATTAAAAATAACTTTTTTATTTATTTAAATAAGATATTCAAATAAGGA
      : :::   ::   : : : ::::     :::: ::::     :: ::::  ::: :: :
_     ACAATATTTTTTCTAAGAGATAA-----TTATATATT-----AATATATATAAAAAAAGG
        310       320            330            340       350

        360       370       380       390       400       410
_     TATTTATTAATTAGCTCGCAAATGGCCAAATAAGAAATATAATATAATATATTATTATAT
      : ::: ::  ::   :    : :      ::    : : ::   :::::: ::::: :
_     TGTTTTTTTTTTTTTTTTTTATTTTT---ATTTTTATTTTATGGTAATATTTTATTTTCC
        360       370       380           390       400       410

        420       430       440       450       460       470
_     ATATTATATATATATATATATATAAATATATTTATAATAATA-ATATAAATAAAGTATAT
       :::: :::: :: :::::   :  :::: :   ::::  :: :::: :  ::  :::::
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           420       430       440       450       460       470
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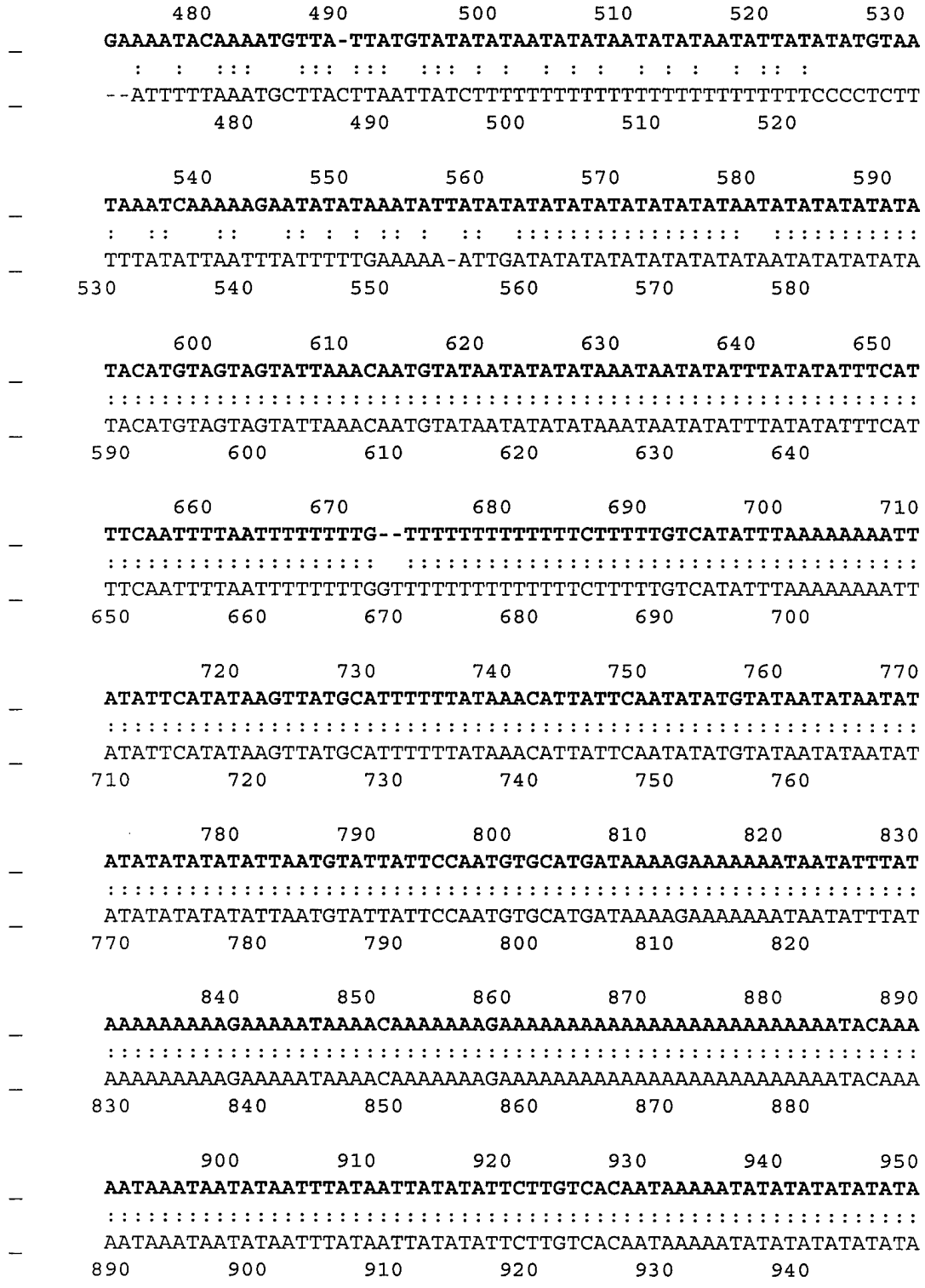
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--ATTTTTAAATGCTTACTTAATTATCTTTTTTTTTTTTTTTTTTTTTTTTTCCCCTCTT
480 490 500 510 520
540 550 560 570 580 590
TAAATCAAAAAGAATATATAAATATTATATATATATATATATATATAATATATATATATA
TTTATATTAATTTATTTTTGAAAAA-ATTGATATATATATATATATATAATATATATATA
530 540 550 560 570 580
600 610 620 630 640 650
TACATGTAGTAGTATTAAACAATGTATAATATATATAAATAATATATTTATATATTTCAT
TACATGTAGTAGTATTAAACAATGTATAATATATATAAATAATATATTTATATATTTCAT
590 600 610 620 630 640
660 670 680 690 700 710
TTCAATTTTAATTTTTTTTG--TTTTTTTTTTTTTCTTTTTGTCATATTTAAAAAAAATT
TTCAATTTTAATTTTTTTTGGTTTTTTTTTTTTTCTTTTTGTCATATTTAAAAAAAATT
650 660 670 680 690 700
720 730 740 750 760 770
ATATTCATATAAGTTATGCATTTTTTATAAACATTATTCAATATATGTATAATATAATAT
ATATTCATATAAGTTATGCATTTTTTATAAACATTATTCAATATATGTATAATATAATAT
710 720 730 740 750 760
780 790 800 810 820 830
ATATATATATATTAATGTATTATTCCAATGTGCATGATAAAAGAAAAAAATAATATTTAT
ATATATATATATTAATGTATTATTCCAATGTGCATGATAAAAGAAAAAAATAATATTTAT
770 780 790 800 810 820
840 850 860 870 880 890
AAAAAAAAAGAAAAATAAAACAAAAAAAGAAAAAAAAAAAAAAAAAAAAAAAATACAAA
AAAAAAAAAGAAAAATAAAACAAAAAAAGAAAAAAAAAAAAAAAAAAAAAAAATACAAA
830 840 850 860 870 880
900 910 920 930 940 950
AATAAATAATATAATTTATAATTATATATTCTTGTCACAATAAAAATATATATATATATA
AATAAATAATATAATTTATAATTATATATTCTTGTCACAATAAAAATATATATATATATA
890 900 910 920 930 940

A. Functional analysis of putative drug resistance and new drug target genes in the heterologous yeast expression system

1. Identification of new drug target genes through complementation analysis in yeast. A single yeast strain with mutations in PDR5/10/SNQ2 has been chosen for this work.

2. Development of new, rapid, high throughput drug screening methods for malaria genes expressed in yeast

This work continues as previously and also includes new initiatives that make use of technology not previously available. The use of the Yeast DNA Microarray in collaboration with the groups of Dr. Maryanne Vahey, Dr. Dennis Kyle and Dr. Keith Martin, WRAIR has greatly facilitated our work and identified new approaches to drug development using this heterologous system. The initial results of this work have been submitted for publication and the ongoing work is summarized below.

(i) Microarray analysis

The majority of the work towards my thesis has involved the analysis of global expression patterns of Saccharomyces cerevisiae when exposed to the antimalarial compound chloroquine. Our laboratory is interested in the mechanisms of drug resistance employed by protozoan parasites with specific interest in the role of membrane transports in resistance. Chloroquine is an important compound for malarial treatment and an understanding of the ways in which organisms respond and develop resistance to this compound is of interest. The choice of yeast as the model system for this analysis is based on several points:

The global expression response to antimalarial compounds has not been investigated in any organism

The yeast system provides a unique combination of tools in the form of a complete genomic sequence with approximately 70% annotation of function and microarray technology that allows the simultaneous observation of all of the Open Reading Frames (ORFs) of the yeast genome

Presence of a network of ATP-binding Cassette (ABC) transporters (pleiotropic drug resistance) with similarity to ABC transporters found in other systems that are involved in Multi-drug resistance phenotypes (see figure 1 for list of yeast ABC transporters)

The availability of yeast strains produced by functional knock-out that are sensitive to compounds of interest

This type of analysis will not only provide a better understanding of how the Pleiotropic Drug Resistance (PDR) network of yeast, and the corresponding ABC-transporters in the parasite system, functions but will also provide leads to additional mechanisms and homologues in the parasite system.

B. Materials and Experimental Design

This study is utilizing the Affymetrix Gene Chip Ye6100 yeast chip system. This system is composed of 5 chips, 1 test chip and 4 yeast ORF chips. The test chip is used for quality control and contains representative ORFs from several organisms and a set of spike controls that are also present on the 4 main yeast ORF chips.

The 4 main chips contain probes for approximately 6200 yeast ORF that cover virtually the entire yeast genome. Each probe set consists of ~20 sets of 25mer oligonucleotides that are exact matches to the genomic sequence and corresponding sets of one base mismatches. The mismatch sets provide controls for background and non-specific hybridization. The resolution range for this assay is 0.1-100 mRNA molecules per cell.

The yeast strains used for this study are the PDR functional knockout YHW1052, which has functional disruptions in 3 ABC-transporters, and the parental wild-type strain YPH499. The specific genotypes for these strains are given in table I.

Three treatment points were selected based on the growth curves depicted in figure 6. The three points increase in severity with increase in number: T1 (2hr-1.5mg/ml), T2 (3hr-2.5mg/ml), T3 (4.5hr-2.5mg/ml). The T1 treatment was selected to examine the expression profile at a point on the growth curve just before the two strains diverged from one another with the expectation that expression levels would already have significant differences. The T3 point was selected to examine the profile under extreme drug stress.

C. Results

The gross global expression patterns observed over the three treatment points for each strain are depicted in figures 7 and 8. These graphs show all ORFs that had a differential of 3-fold in expression levels comparing drug treated to control. These ORFs are divided into 12 functional families by their annotations in the Saccharomyces Genome and MIPS Genome Databases. It is interesting to note that although roughly 70% of the yeast database is annotated with either similarity or direct functional data the category of Unknown Function (UNK) still ranks as the top group for expression response to the drug treatment as compared to control. This has also been observed in other microarray studies such as those conducted by the Jelinsky and Samson.

Comparing the two profiles there is a significant difference in expression response between the two strains. The peak in expression response, measured by number of ORFs having a 3-fold differential, for the wild-type parental strain (YPH499) is in T2 while

that of the functional knockout (YHW1052) occurs during T3. The majority of the this peak response for the YHW1052 strain is a decrease in expression as compared to the control and the cells appear very unhealthy on visual inspection. It is possible that the expression profile for the functional knockout strain in T3 is largely the result of cellular death processes.

Another indication of the differences in expression profiles between the two strains is the small number of high response ORFs that are in common for the two strains. Both strains have more than 80 ORFs responding with a 6-fold change but only 11 of these are shared. Once again roughly half of these ORFs are of unknown function.

Challenges associated with this type of analysis are data handling and the selection of specific targets for further study. Initial targets that we have selected are listed in figure 9. These include the ABC-transporters of the PDR network and two members of the Major Facilitator Super family (MFS) of small molecule transporters.

The PDR transports were selected based on the interests of our lab as a whole and the relation of this network of transporters with several aspects of drug resistance. Observations made by our laboratory and that of Karl Kuckler's, the source of our pdr yeast strains, indicated overlapping function and substrate specificity for members of the pdr network. The current study provides an opportunity to investigate the expression patterns that associated with this phenomenon and to more clearly elucidate the interactions of these transporters.

The MFS transporter SIT1, an iron siderophore transporter, has been selected based on the magnitude of its expression in the two strains and its status as one of the shared ORFs in the expression responses of the strains 2 (Lesuisse et al 1998). YOR273C, the other MFS member, was selected based on the support of our expression data by an independent functional screen performed by Delling et al. in which a yeast genomic S.c. library was screen for conference of resistance to quinoline ring-containing antimalarial compounds (Delling,. et al 1998)

In figure 9 the largest differential for each of the selected ORFs is reported. In the wild-type strain (YPH499) PDR5 has a small but significant increase compared to control in the treated sample. The PDR5 gene is the member of the PDR network that shows the greatest similarity to the PfMDR1 gene of Plasmodium falciparium. In the case of the functional knockout strain (YHW1052) there are significant increases in three members of the PDR network family, PDR12, PDR15, and YOR1, in response to the removal of PDR5, PDR10, and SNQ2. This observation appears to further support the hypothesis that these transporters have overlapping responses and substrate specificity.

YOR273C shows significant expression in both strains and this expression is supported by Northern Slot analysis depicted in figure 10.

D. Summary and Future Directions

Chloroquine treatment affects the expression of >200 ORFs in each strain

Gene expression profile is dependent on genetic background; specifically the functional knockouts have a significant impact

Expression of PDR-related transporters supports the predicted roles and the hypothesis of overlapping response and substrate specificity

There are several indications for a role of YOR273C in responses to quinoline ring compounds

Northern Slot analysis confirms the chip data on YOR273C

Northern Slot analysis will be continued to confirm chip data on other ORFs of interest. Further analysis of the array data will be conducted using cluster and temporal approaches in order to discover further associations and patterns. Our data will also be compared with other published and available array data in order to discern general stress responses and other general response phenomenon. Overexpression and Knockout experiments are planned with selected targets. Additional knockout strains for PDR transports are in hand and analysis of these is underway.

In addition we are interested in conducting an additional chip analysis of the original strains with an another compound (FK506, fluconazole, ketoconazole, rhodamine6G) as yet to be determined.

(ii) Confirmation of Complementation and Mating Phenotype

Recently we demonstrated that expression of *PfMDR1* in yeast deficient for *ste6*, resulted in complementation of the mating phenotype conferred by the native STE6 protein in yeast (Volkman, *et al.*, *PNAS* (95) **92**, 8921]. Ruetz et al. [*PNAS* (96) **93**, 9942] reported complementation of *ste6* with *PfMDR1*, and that expression of *PfMDR1* conferred drug resistance in yeast for quinine, quinacrine, mefloquine and halofantrine. The observation that *PfMDR1* expression conferred drug resistance in yeast is different than our findings that *PfMDR1* expression is associated with increased drug sensitivity in yeast. Recently these data of Ruetz et al. have been retracted [*PNAS* (99) **96**, 1810], citing that *ste6* sequences were identified in yeast transformants believed to contain *PfMDR1*. Because of this report we wanted to confirm our original findings, that expression of *PfMDR1* in yeast deficient for *ste6* restores a mating phenotype, and these data are reported here. The goal of these experiments was to (1) confirm the previously observed mating phenotype; (2) demonstrate that mating is due to the presence of *PfMDR1*; and (3) show that mating is not due to the presence of *ste6*. Similar experiments are independently being conducted in other laboratories to confirm these results.

Two independently derived plasmid constructs containing the *PfMDR1* gene were tested (pY*PfMDR1*-1 and pY*PfMDR1*-2). As controls, the same plasmid containing either no insert (pY) or the *ste6* gene (pY*ste6*) was used. Yeast strains used were the *ste6*-deficient (*ste6*) strain SM1563 [**a** *trp*1 *leu*2 *ura*3 *his*4 *can*1 *ste6*::*LEU2*) into which plasmids were transformed, and the MAT strain SM1068 [*lys*1] to test the ability of the transformants to confer a mating phenotype. Three independent mating assays were performed with three single colonies from new transformation experiments for each of the two *PfMDR1* plasmids. Mating assays were performed by mixing 10^7 MAT**a** cells with 10^8 MAT cells, and plating the mixture on SD plates. The number of diploids formed after two days was counted with the data from three mating assay shown in **Table II**. These data confirm that yeast containing both of the *PfMDR1* plasmids were able to complement the STE6 phenotype and restore the ability of yeast deficient for *ste6* to mate.

It was observed that on average, only approximately one out of every ten to fifty transformants resulted in successful complementation of mating phenotype (data not shown). The reason for this is not known, but presumably plasmid loss or rearrangement results in the loss of *PfMDR1* expression in these cells. Freshly transformed cells were more likely to yield transformants that complemented *ste6*, and for these experiments, three of five colonies isolated for each plasmid (pY*PfMDR1*-1 or pY*PfMDR1*-2) successfully mated. Experiments performed in our laboratory used yeast strains with distinct genetic backgrounds, and different plasmid constructs expressing *PfMDR1* that were derived independently from those used in the work by Ruetz et al. When plasmids containing *PfMDR1* sequences received from Dr. Phillipe Gros (pVT-*PfMDR*) were tested in our yeast assay system, these transformants did not restore a mating phenotype (**Table II**).

Table II: Summary of Mating Phenotype for Yeast Transformed with *PfMDR1*

Yeast Transformant	I	II	III	IV
pY	0	0	0	0
pY*ste6*	>2000*	>1500*	>2000*	>2000*
pY*PfMDR1*-1.1	207	149	115	79
pY*PfMDR1*-1.2	241	139	228	
pY*PfMDR1*-1.3	189	186	153	
pY*PfMDR1*-2.1	95	82	196	
pY*PfMDR1*-2.2	133	114	134	
pY*PfMDR1*-2.3	119	109	177	

Yeast Transformant	IV	V
pY	0	0
pY*ste6*	>2000*	>2000*
pY*PfMDR1*	79	101
pVT-*PfMDR*-1	0	0
pVT-*PfMDR*-2	0	0

Yeast transformed with *PfMDR1* plasmid conferred a mating phenotype. MAT**a** yeast deficient for *ste6* (SM1563) were transformed with pY*PfMDR1*, and three separate transformants for each of the two independently derived yeast expression plasmids containing *PfMDR1* (pY*PfMDR1*-1 and pY*PfMDR1*-2) were analyzed. In three separating mating assays, a total of 10^7 MAT**a** cells and 10^8 MAT cells were incubated and the number of diploids recovered for each experiment (I-III)are reported.

Additional experiments using two pVT-PfMDR plasmids (Ruetz et al) were performed (IV-V). The number of diploids for the *ste6* control was estimated by plating a dilution of the mixture.

To test if the observed mating phenotype was due to the presence of the *PfMDR1* gene, and to demonstrate that the native *ste6* gene is not present in these yeast transformants, experiments using the polymerase chain reaction (PCR) were performed using gene specific primers. This analysis was performed both on total DNA derived from yeast transformants that had successfully mated, as well as on plasmid DNA recovered from bacteria transformed with a sample of this total DNA. Primer sequences for *ste6* were derived from nucleotides 755-780 and 2069-2095 and amplified a product of approximately 1340 nucleotides, while primer sequences for *PfMDR1* were derived from nucleotides 510-534 and 1462-1487 and amplified a product of approximately 980 nucleotides (**Figure 1**). These data demonstrate that DNA derived from yeast that conferred a mating phenotype contained *PfMDR1* sequences for yeast transformed with either pY*PfMDR1*-1 (see Figure 1A, lanes 2 and 3) or pY*PfMDR1*-2 (see Figure 1A, lanes 4 and 5), and did not contain contaminating *ste6* sequences. Similarly, plasmids derived from bacteria transformed with these DNA samples contained the expected *PfMDR1* sequences from yeast transformed with either pY*PfMDR1*-1 (see Figure 1B, lanes 3-9) or pY*PfMDR1*-2 (see Figure 1B, lanes 10-13), and did not contain *ste6* sequences. These data demonstrate that yeast transformed with *PfMDR1* that conferred a mating phenotype contain *PfMDR1*, but not *ste6*. Together these data demonstrate that yeast deficient for *ste6* that are transformed with *PfMDR1* restore a mating phenotype, and that this mating phenotype is due to the presence of *PfMDR1* and not contaminating *ste6* sequences.

(7) Key Research Accomplishments

Development of the Serial Analysis of Gene Expression (SAGE) system for *Plasmodium falciparum*

Analysis of 5' UTR of *Plasmodium falciparum* genes

Development of Yeast Microarray

(8) Reportable Outcomes

Manuscripts

Golightly LM, Mbacham W, Daily J, Wirth DF. 3' UTR elements enhance expression of Pgs28, an ookinete protein of *Plasmodium gallinaceum*. Molec Biochem Parasit. 1999; in press.

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Wirth DF. Invited Speaker. Gordon Research Conference, June 20-25, 1999, Newport, R.I.

(9) CONCLUSIONS

Malaria represents a major and increasing threat to the U.S. Military. Many of the sites of current or potential U.S. Military involvement are endemic for malaria and in several sites, multidrug resistant *P. falciparum* represents a major problem especially for non-immune military personnel. Current drugs available to the U.S. Military are quickly losing their effectiveness because of emerging and spreading drug resistance. This work is directed both at identifying new drugs and drug targets, but equally importantly toward an understanding of drug resistance mechanisms with the goal of preventing or overcoming drug resistance in the malaria parasite.

A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs based on an understanding of the parasite biology are key elements in this new strategy. Clearly the development of a new antimalarial will require both basic and applied research working in concert with one another.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. Progress has been made in several key areas. During this year we have tried new technical approaches to address the key goals of this work. These technical approaches were not available at the time of the original plan and are based on the rapidly evolving genome projects, including the completion of the yeast genome sequence and the development of the Plasmodium falciparum genome project. We have used these advances both in developing methods for understanding gene expression in response to drug treatment and in the future hope to use these methods to identify new drug targets.

(10) REFERENCES

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Table I

Strains	Genotypes
YPH 499	MATa; ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52
YHW 1052	Δpdr5::TRP1, Δsnq2::hisG, Δpdr10::hisG
YHW 10515K	Δpdr5::TRP1, Δpdr10::hisG, Δpdr15::loxP-KanMx-loxP
YRP3	Δpdr5::TRP1, Δsnq2::hisG, Δste6::hisG, Δerg6::LEU2
YYM3	Mat alpha, Δpdr5::TRP1, Δsnq2::hisG
YRP2	Δerg6::LEU2
YYM4	Δpdr5::TRP1, Δsnq2::hisG
YYM5	Δsnq2::hisG
YKKA-7	Δpdr5::TRP1

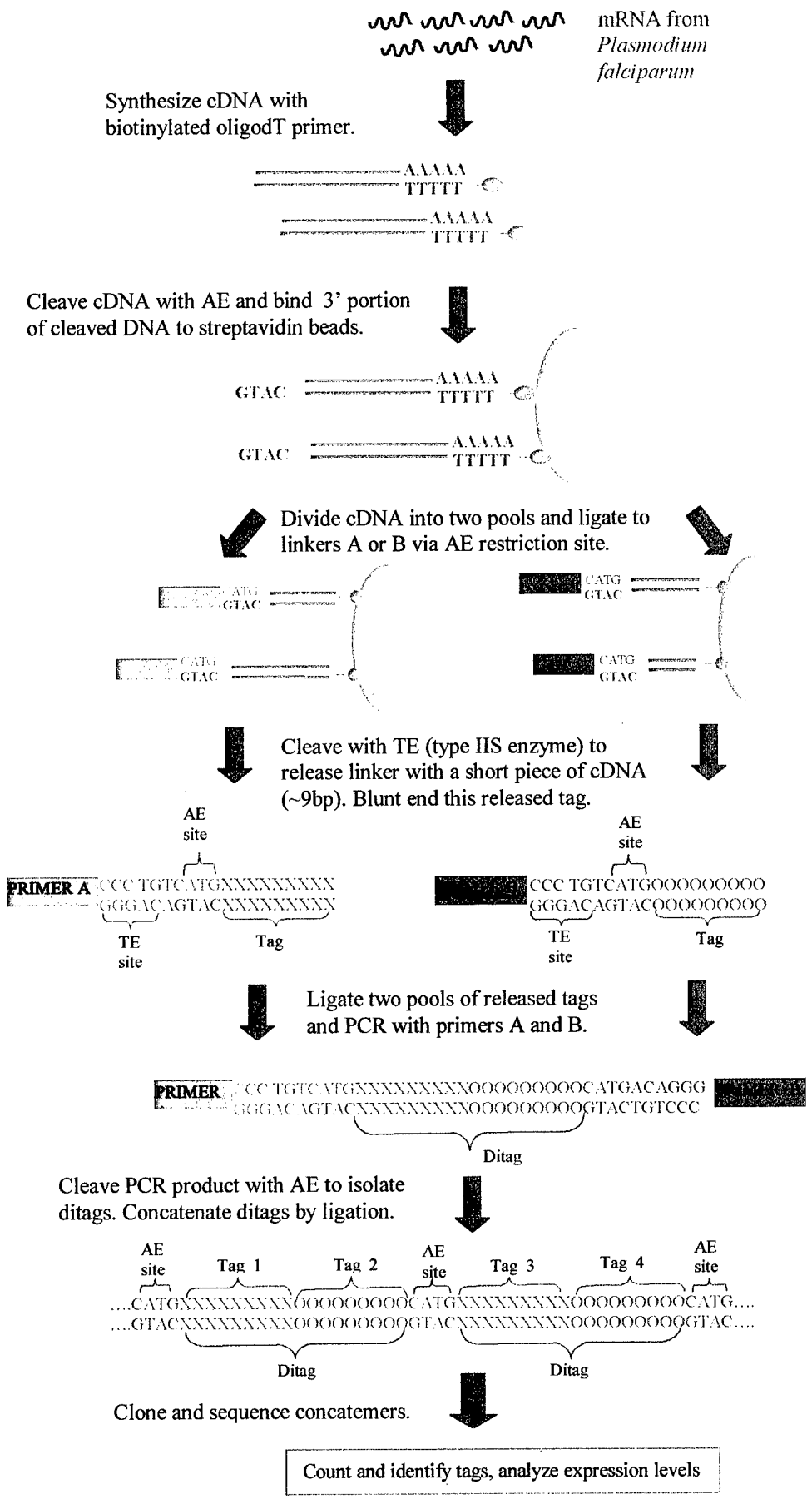


Fig. 1: Schematic of SAGE. The anchoring enzyme (AE) is NlaIII and the tagging enzyme (TE) is BsmFI. Sequences in green represent *P.falciparum* derived sequence; sequences in light blue and purple represent primer derived sequence. X and O indicate nucleotides of different tags.

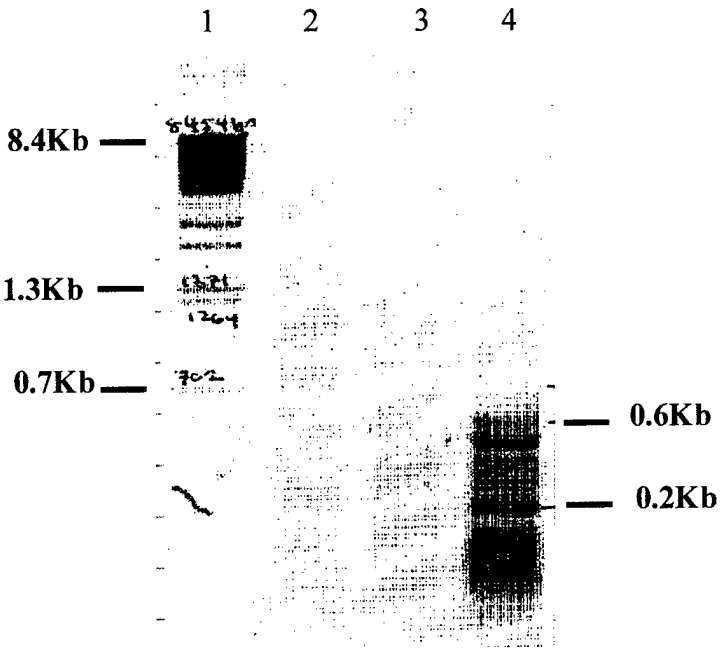


Fig 2: cDNA from *P.falciparum*
Lane 1: lambda BstEII marker
Lane 2: parasite cDNA
Lane 3: parasite cDNA digested with NlaIII
Lane 4: pBR322 MspI marker

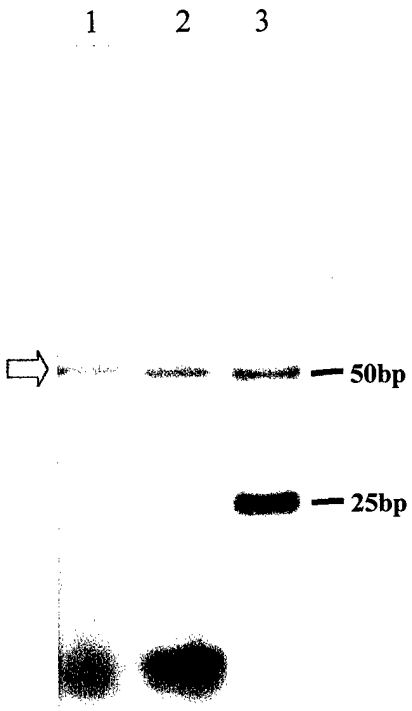


Fig 3: Autoradiograph of blunt ended tags
Lane 1: blunt ended tags from pool A
Lane 2: blunt ended tags from pool B
Lane 3: marker
Red arrow marks position of tags that were blunt ended with klenow in the presence of radiolabelled dATP.

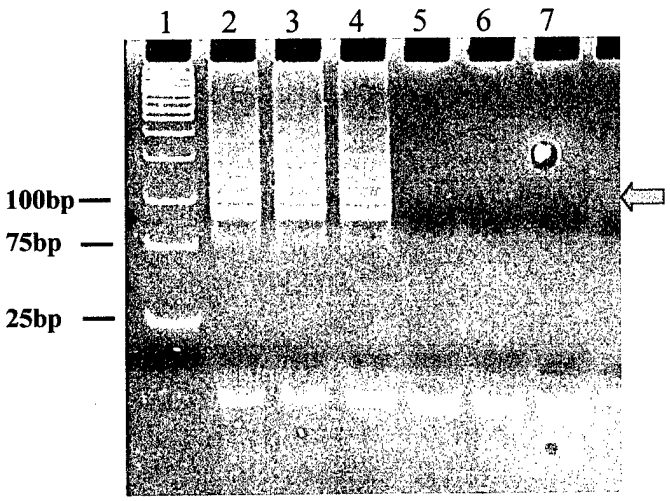


Fig 4: Polyacrylamide gel of PCR reactions.
Lane 1: Marker
Lanes 2-4: blunt-ended tags + T4 DNA ligase
Lanes 5-7: blunt-ended tags - T4 DNA ligase
Red arrow marks the position of the 102bp amplification product

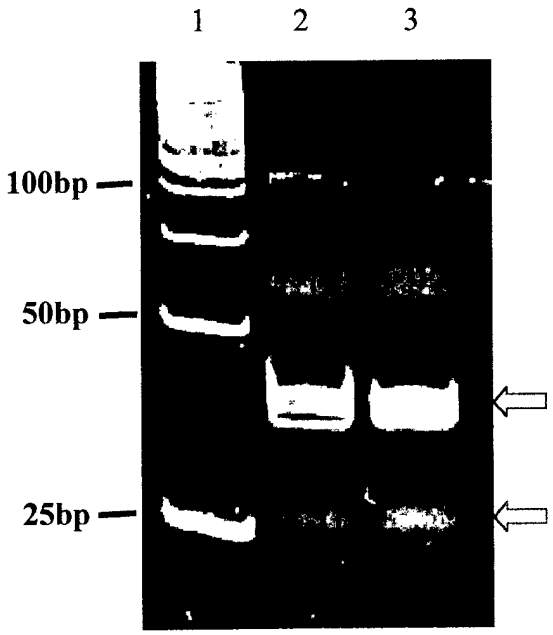


Fig 5: Polyacrylamide gel of released 26bp ditags
Lane 1: marker
Lane 2-3: 102bp product digested with NlaIII
Red arrow marks the position of the released 26mer ditags
Blue arrow marks the position of released linkers

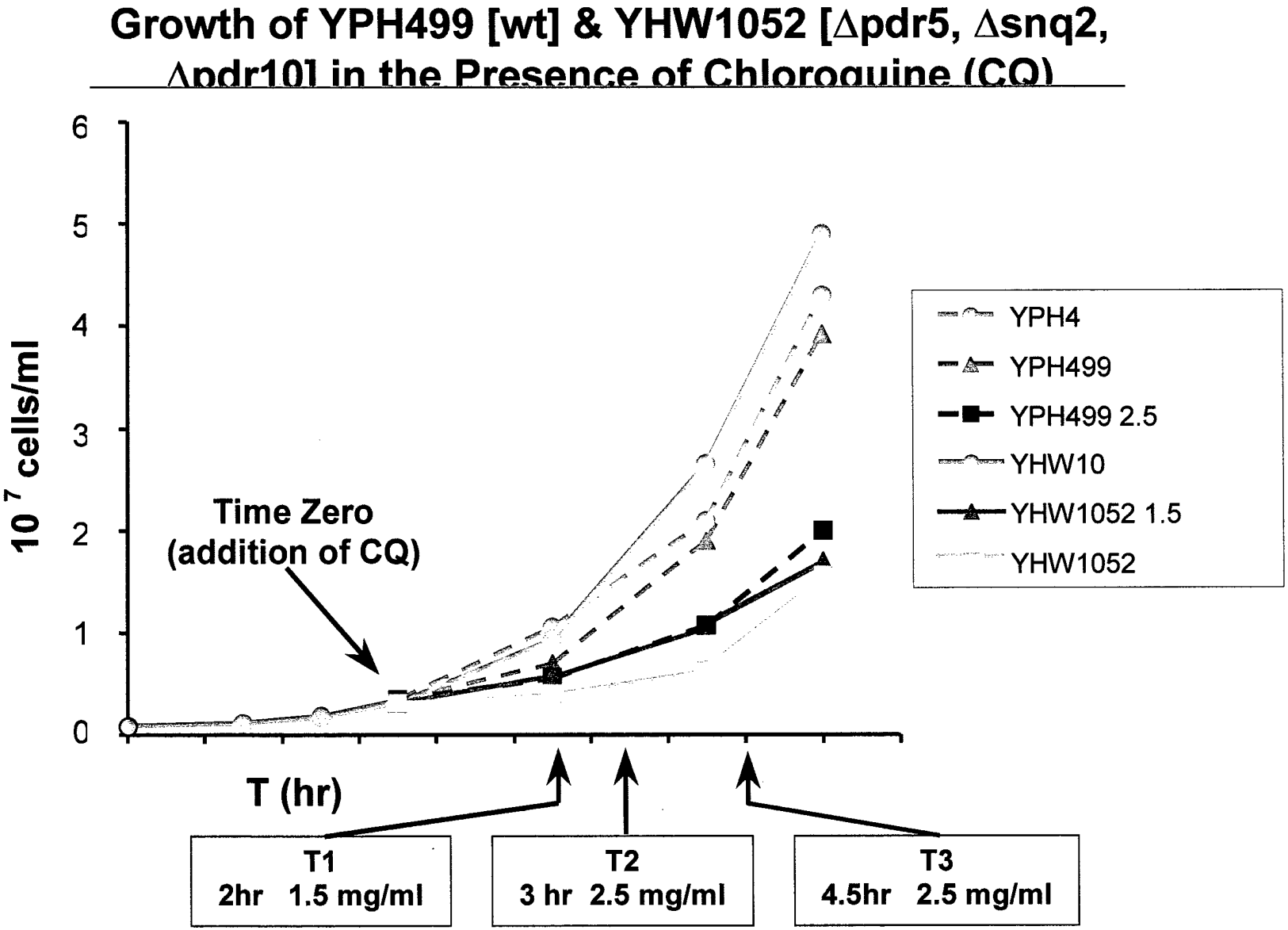

Growth of YPH499 [wt] & YHW1052 [Δpdr5, Δsnq2, Δpdr10] in the Presence of Chloroquine (CQ)
10 7 cells/ml
0
1
2
3
4
5
6
Time Zero
(addition of CQ)
T (hr)
YPH4
YPH499
YPH499 2.5
YHW10
YHW1052 1.5
YHW1052
T1
2hr 1.5 mg/ml
T2
3 hr 2.5 mg/ml
T3
4.5hr 2.5 mg/ml

Figure 6

YHW1052 ORFs with >3-Fold Difference as Compared to No-Drug Control

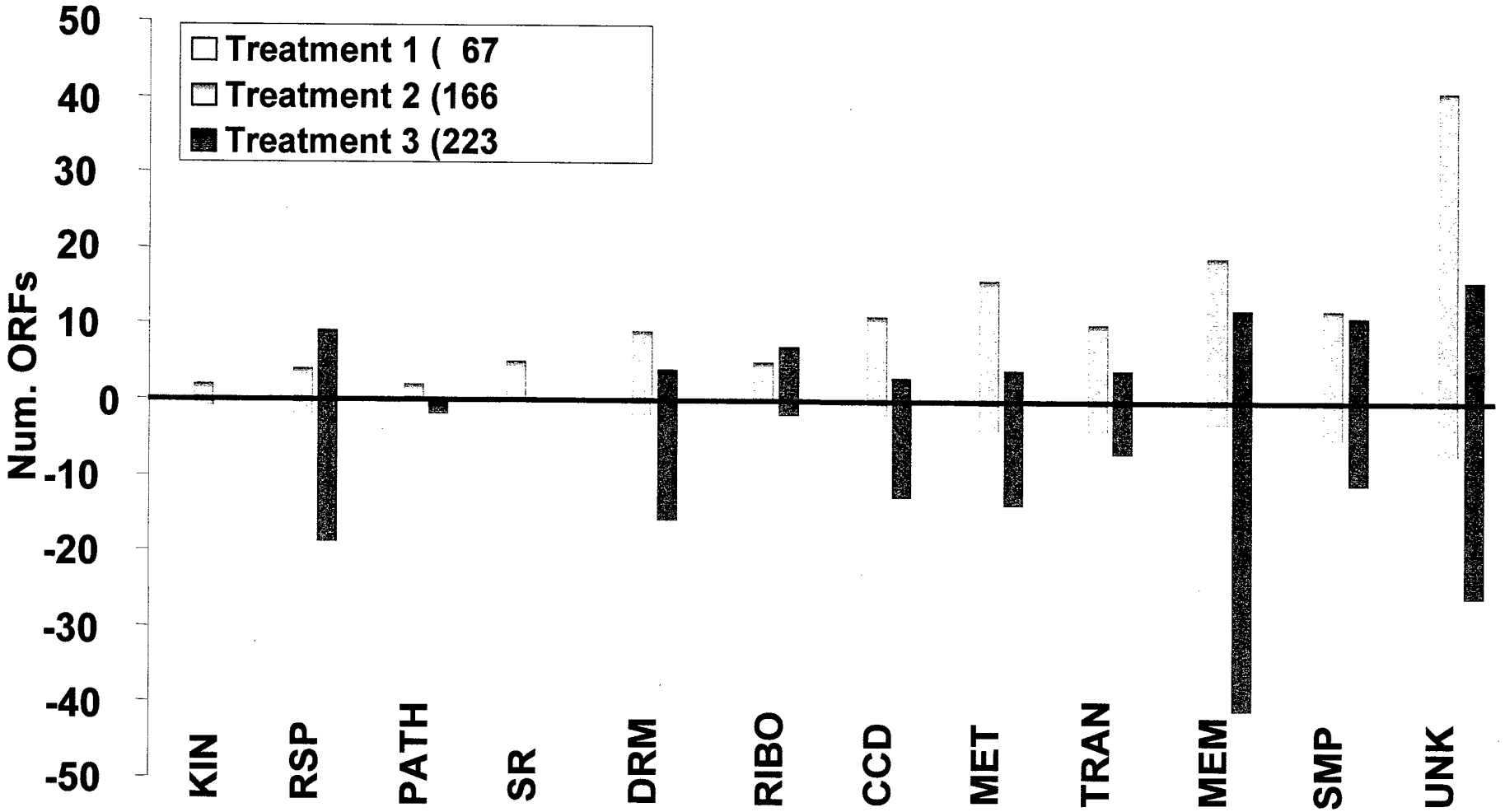


Figure 7

YPH499 ORFs with >3-Fold Difference as Compared to No Drug Control

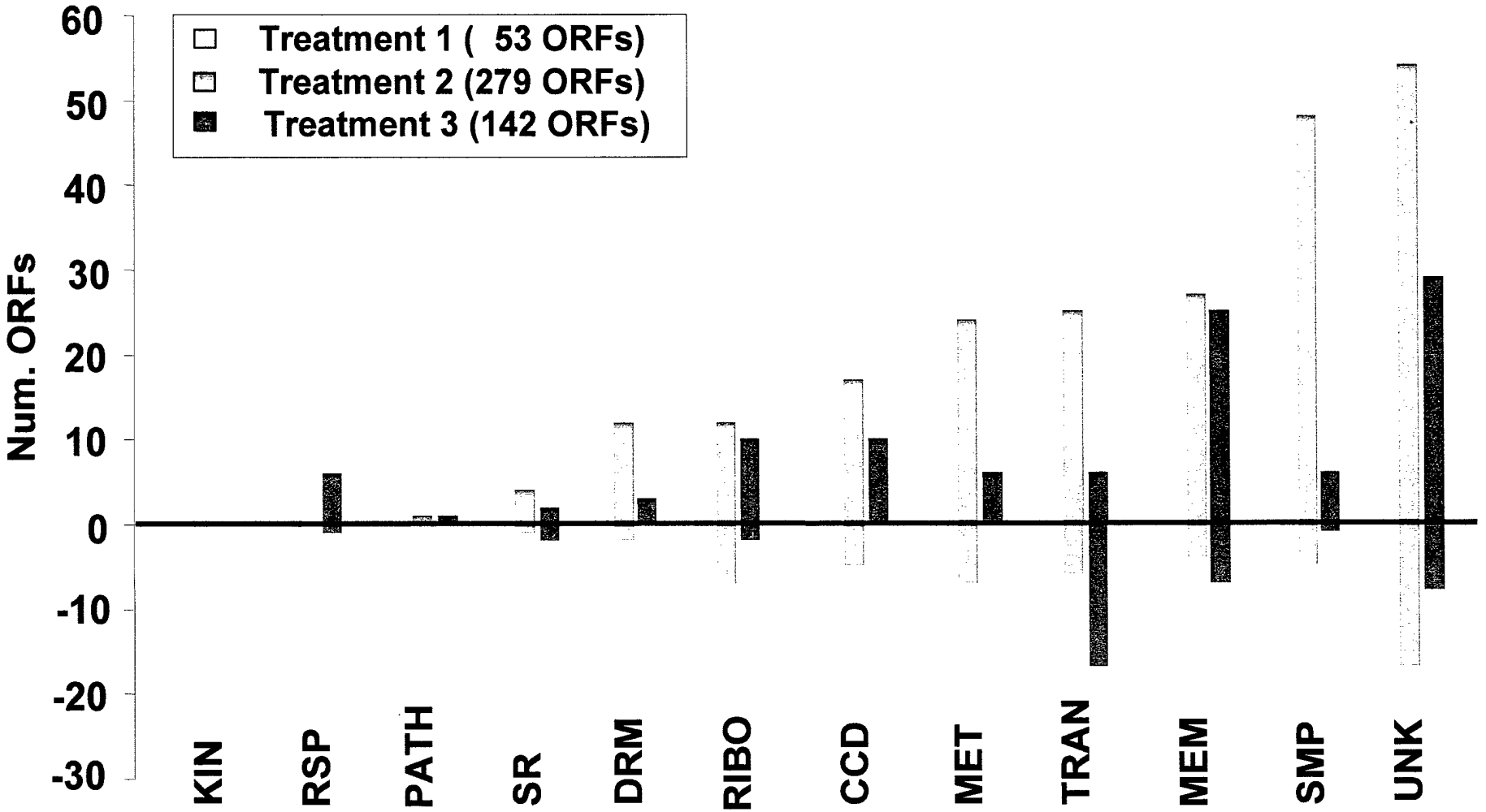


Figure 8

Response of Membrane Transporters to CQ Treatment
(Maximum Differential Observed Comparing Treated to Control)

		YPH 499	YHW1052
YDR011W	*SNQ2*	ND	KO
YOR153W	*PDR5*	1.7	KO
YOR328W	*PDR10*	ND	KO
YIL013C	*PDR11*	ND	-2.1
YPL058C	*PDR12*	ND	2.1
YDR406W	*PDR15*	ND	3.1
YGR281W	*YOR1*	-4.6	3.2
YDR135C	*YCF1*	ND	ND
YOR273C		3.2	3.6
YEL065W	*SIT1*	6.0	-16.7

Figure 9

Northern Slot Blot Analysis of YOR273C for TP 3

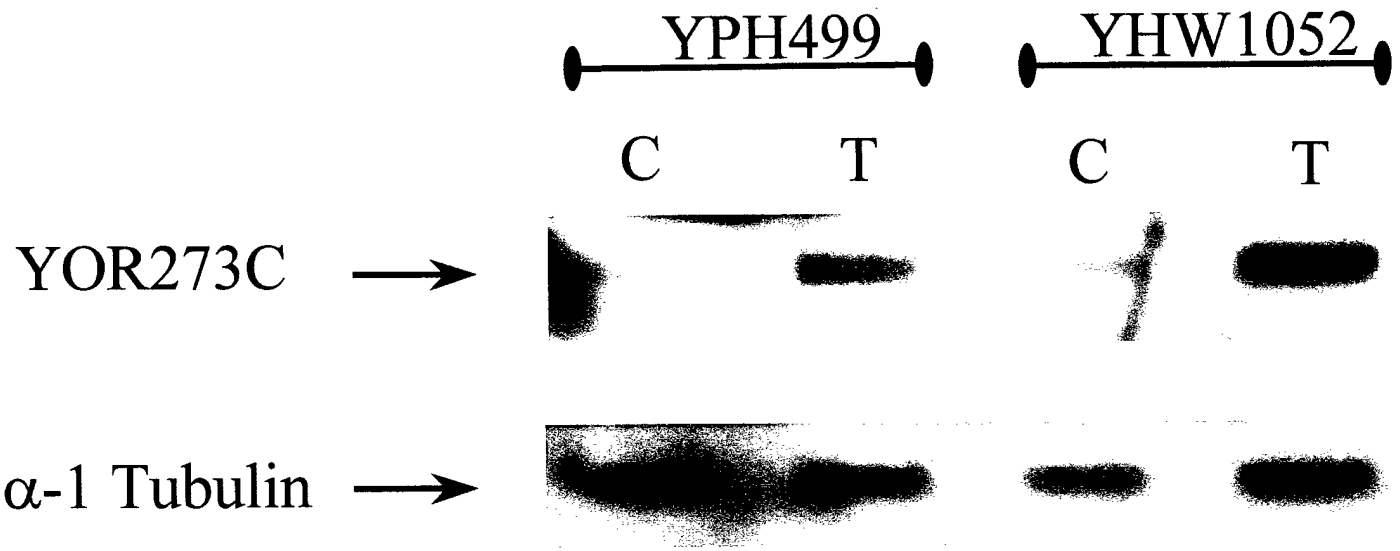
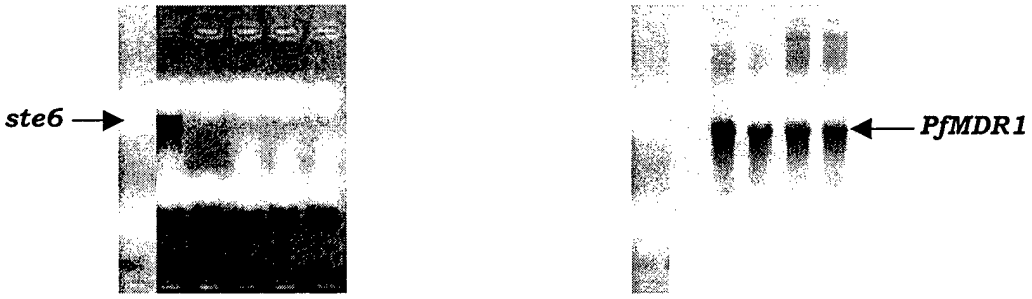


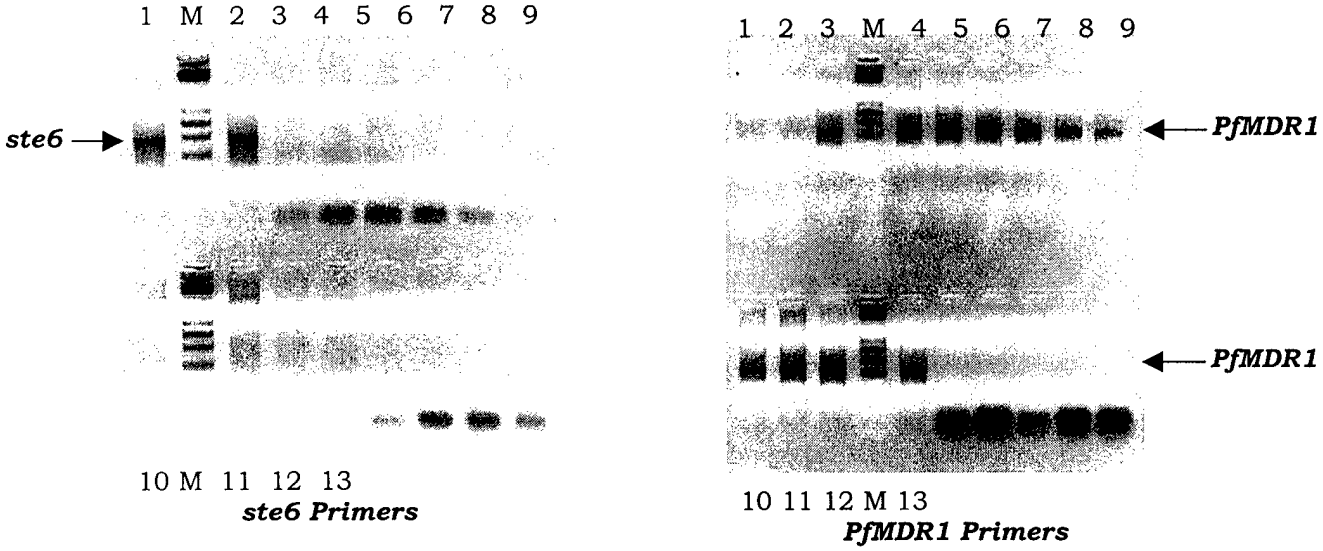
Figure 10

Figure 11: Detection of *PfMDR1* in Yeast Transformants with Mating Phenotype

A. PCR Analysis of Total DNA from Yeast Transformants



B. PCR Analysis of Plasmids Recovered in *E. coli* from Yeast Transformants



Yeast transformed with pY*PfMDR1* that conferred a mating phenotype contained the *PfMDR1*, but not the *ste6* gene. Total DNA isolated from yeast transformed with either pY (A1), pY*ste6* (A2) or pY*PfMDR1*-1 (A3-A4) or pY*PfMDR1*-2 (A5-A6) plasmid was used as template for amplification of gene specific sequences using the polymerase chain reaction (PCR). This total DNA was also used to transform *E. coli* to recover plasmid DNA, which was subsequently used as template in a similar PCR experiment using gene specific primers. Samples of the resulting PCR analysis were resolved in agarose with a standard marker (M) and visualized after incubation with ethidium bromide. Independent bacterial clones transfomed with pY*ste6* (B1-B2) or pY*PfMDR1*-1 (B3-B9) or pY*PfMDR1*-2 (B10-B13) were used for this analysis.